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(54) Title: PEPTIDES AND COMPOUNDS THAT BIND TO THE IL-1 RECEPTOR

(57) Abstract

Peptides that bind to the interleukin-1 type I receptor (IL-1RtI) can be used to assay the amount of IL-1R, or an IL-1R agonist or antagonist, in a sample and comprise a sequence of amino acids selected from the group consisting of (1) WXXXGZ$_1$W where Z$_1$ is L, I, A, or Q; (2) XXQZ$_5$YZ$_6$XX where Z$_5$ is P or Aze where Aze is azetidine; and Z$_6$ is S, A, V, or L; and (3) Z$_{23}$NZ$_{24}$SZ$_{25}$Z$_{26}$Z$_{27}$Z$_{28}$Z$_{29}$Z$_{30}$L where Z$_{23}$ is D or Y; Z$_{24}$ is D or S; Z$_{25}$ is S or W; Z$_{26}$ is S or Y; Z$_{27}$ is D or V; Z$_{28}$ is S or W; Z$_{29}$ is F or L; and Z$_{30}$ is D or L; and where each amino acid is indicated by standard one letter abbreviation; and each X can be selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids.

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PEPTIDES AND COMPOUNDS THAT BIND TO THE IL-1 RECEPTOR

CROSS-REFERENCE

This application is a continuation-in-part of U.S. Patent application No. 08/190,788, filed on February 2, 1994, which is incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

The present invention provides peptides and compounds that bind the interleukin 1 receptor (IL-1R), methods for assaying interleukin 1 (IL-1), and methods for inhibiting the binding of IL-1 to the IL-1R. The invention has application in the fields of biochemistry and medicinal chemistry and particularly provides IL-1 antagonists and agonists for use in the treatment of human disease.

IL-1 is a polypeptide hormone, a cytokine, that exists in various forms, the genes for two of which, IL-1α and IL-1β, have been cloned. Unless otherwise noted, "IL-1" refers to either or both IL-1α and IL-1β. These two genes are both located in chromosome 2; each gene contains 7 exons, and the two genes are homologous in a region of the sixth exon. Both IL-1α and IL-1β initially exist as 31 kD precursors but are processed by proteases to produce the amino terminus of the 17.5 kD mature proteins. Receptors for IL-1 recognize the α and β forms, and both forms have similar biological properties. *See* Dinarello (1991) *Blood* 77(8):1627-1652, incorporated herein by reference.

The biological properties of IL-1 include mediating many immunological and inflammatory responses to infection and tissue injury. Because of the role of IL-1 in these important processes, the therapeutic benefits of IL-1 and derivatives of IL-1 have been extensively studied. *See* U.S. Patent Nos. 5,075,288 and 5,077,219, incorporated herein by reference. Inappropriate production or response to IL-1 plays a role in many chronic inflammatory diseases, such as rheumatoid

arthritis, osteoarthritis, psoriasis, inflammatory bowel disease, encephalitis, glomerulonephritis, and respiratory distress syndrome. *See* Bender and Lee (1989) *Ann. Rep. Med. Chem.* 25:185-193; and U.S. Patent No. 5,075,222, particularly columns 1 to 3, each of which is incorporated herein by reference.

Because of the important biological properties of IL-1, IL-1 inhibitors have been extensively studied, as reviewed in Larrick (1989) *Immunol. Today* 10 (2):61-66, incorporated herein by reference. IL-1 inhibitors include the naturally occurring IL-1ra protein and soluble IL-1 receptor, as well as derivatives of IL-1α and IL-1β produced by recombinant DNA technology, as discussed in Dinarello, *supra*. *See also* PCT patent publication Nos. 91/08285, published June 13, 1991, and 91/02127, published November 14, 1991, incorporated herein by reference.

In similar fashion, scientists have studied the IL-1R, as reviewed in Dower and Urdal (1987) *Immunol. Today* 8(2):46-51, incorporated herein by reference. Two distinct naturally occurring types of the IL-1R are known to exist, and the corresponding genes have been cloned and expressed, as reported in Dower *et al.*, (1990) *J. Clin. Immunol.* 10 (6):289-299; PCT patent publication No. 91/00742; U.S. Patent No. 4,968,607, and McMahon *et al.*,(1991) *EMBO J.* 10(10):2821-2832, each of which is incorporated herein by reference. The type I receptor (IL-1RtI) is 80 kD in size, while the type II receptor (IL-1RtII) is 60 kD in size. A number of studies regarding whether IL-1RtI and IL-1RtII have different affinities for ligands have been conducted; *see* Slack *et al.* (1993) *J. Biol. Chem.* 268:2513-2524 and Hannum *et al.*, (1990) *Nature* 343:336-340.

The availability of cloned genes for IL-1RtI and IL1RtII, including a soluble IL-1RtI derivative, facilitates the search for agonists and antagonists of these important receptors. The availability of the recombinant receptor protein allows the study of receptor-ligand interaction in a variety of random and semi-random peptide diversity generation systems. These systems include the "peptides on plasmids"

system described in U.S. Patent No. 5,270,170, the "peptides on phage" system described in U.S. patent application Serial No. 718,577, filed June 20, 1991, and in Cwirla *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382, and the "very large scale immobilized polymer synthesis" system described in U.S. Patent No. 5,143,854; PCT patent publication No. 90/15070, published December 13, 1990; U.S. patent application Serial No. 624,120, filed December 6, 1990; Fodor et al., February 15, 1991, *Science* 251:767-773; Dower and Fodor (1991) *Ann. Rep. Med. Chem.* 26 :271-180; and U.S. patent application Serial No. 805,727, filed December 6, 1991; each of the foregoing patent applications and publications is incorporated herein by reference.

There remains a need, however, for compounds that bind to or otherwise interact with the IL-1R, both for studies of the important biological activities mediated by this receptor and for treatment of disease. The present invention provides such compounds.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides peptides that bind to IL-1RtI. These peptides generally comprise a molecular weight less than about 3000 Daltons, a binding affinity to an interleukin type I receptor as expressed by an IC_{50} standard of no more than about 2.5 mM. Further, the binding of the peptide to the IL-1 receptor is competitively inhibited by a second peptide of seven to forty amino acids in length, which comprises a core sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; and Z_6 is S, A, V, or L; or a sequence of amino acids $WXXXGZ_1W$ where each amino acid is indicated by standard one letter abbreviation; each X can be selected from any one of the 20 genetically coded L-amino acids; and Z_1 is L, I, A, or Q.

In a further embodiment, the peptides are seven to forty or more amino acid residues in length, preferably seven to twenty-five amino acid residues in length, and comprise a

core sequence of amino acids $WXXXGZ_1W$ where each amino acid is indicated by the standard one letter abbreviation; each X can be selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; and Z_1 is L, I, A, or Q. More preferably, the core sequence of amino acids will comprise WZ_2XXGZ_1W where X can be selected from any one of the 20 genetically coded L-amino acids; Z_1 is L, I, A, or Q; and Z_2 is D, G, N, S, or T. In a more preferred embodiment, the core sequence of amino acids will comprise $WZ_2Z_3Z_4GZ_1W$ where Z_1 is L, I, A, or Q; Z_2 is D, G, N, S, or T; Z_3, is D, E, H, M, N, Q, R, S, T, or V; and Z_4 is A, D, F, H, K, N, Q, R, T, or Y. Most preferably, the core sequence of amino acids comprise $WZ_2Z_3Z_4GZ_1W$ where Z_1 is L or I; Z_2 is D, S, or T; Z_3 is D, E, or T; and Z_4 is D, H, N, R, or T. An especially preferred embodiment is one having the substitution patterns just described, but comprising 8, 10, or 12 amino acid residues. An especially preferred peptide has the sequence SWDTRGLWVE.

According to another embodiment, the peptides are eight to forty or more amino acid residues in length, preferably eight to twenty-five amino acid residues in length, and comprise a core sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids or unnatural amino acids; Z_5 is P or Aze where Aze (or J) is azetidine; and Z_6 is S, A, V, or L. More preferably, the sequence of amino acids will comprise $Z_7XQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; Z_6 is S, A, V, or L; and Z_7 is Y, W, or F. In a more preferred embodiment, the core sequence of amino acids will comprise $Z_7Z_8QZ_5YZ_6Z_9Z_{10}$ where Z_5 is P or Aze where Aze is azetidine, also represented as "J"; Z_6 is S, A, V, or L; Z_7 is Y, W, or F; Z_8 is E, F, V, W, or Y; Z_9 is M, F, V, R, Q, K, T, S, D, L, I, or E; and Z_{10} is E, L, W, V, H, I, G, A, D, L, Y, N, Q or P. More preferably, Z_9 is V, L, I, or E; and Z_{10} is Q or P. Most preferably, the core peptide will comprise a sequence of amino acids $Z_{11}Z_7Z_8QZ_5YZ_6Z_9Z_{10}$, where Z_8 is Y, W or F; and Z_{11} is V, L, I, E, P, G, Y, M, T, or D.

Preferred peptides include FEWTPGYWQPYALPL, FEWTPGYWQJYALPL, FEWTPGWYQPYALPL, FEWTPGWYQJYALPL, FEWTPGYYQPYALPL and FEWTPGYYQJYALPL.

An especially preferred embodiment is one having the substitution patterns just described, but comprising 21 amino acid residues. Particularly preferred is the peptide comprising the sequence of amino acids $Z_{12}Z_{13}Z_{14}Z_{15}Z_{16}Z_{17}Z_{18}Z_{19}Z_{20}Z_{21}Z_{22}Z_{11}Z_7Z_8QZ_5YZ_6Z_9Z_{10}L$, where Z_8 is Y, W or F; Z_{11} is V, L, I, E, P, G, Y, M, T, or, D; Z_{12} is A, D, E, F, G, K, Q, S, T, V, or Y; Z_{13} is A, D, G, I, N, P, S, T, V, or W; Z_{14} is A, D, G, L, N, P, S, T, W, or Y; Z_{15} is A, D, E, F, L, N, R, V, or Y; Z_{16} is A, D, E, Q, R, S, or T; Z_{17} is H, I, L, P, S, T, or, W; Z_{18} is A, E, F, K, N, Q, R, S, or Y; Z_{19} is D, E, F, Q, R, T, or W; Z_{20} is A, D, P, S, T, or W; Z_{21} is A, D, G, K, N, Q, S, or T; and Z_{22} is A, E, L, P, S, T, V or Y. More preferably, Z_8 is Y, W or F; Z_{11} is V, L, I, E, P, G, Y, M, T, or, D; Z_{12} is D, E, Q, S, T, V, or Y; Z_{13} is A, D, G, I, N, S, T or V; Z_{14} is A, G, L, N, P, S, T, or Y; Z_{15} is D, E, F, L, V, or Y; Z_{16} is D, R, S or T; Z_{17} is H, P, S, or W; Z_{18} is E, F, N, R, Q, or S; Z_{19} is D, E, F, Q, or W; Z_{20} is S, T, or W; Z_{21} is D, G, K, N, Q, S, or T; and Z_{22} is A, E, P, S, or Y.

Particularly preferred peptides include TANVSSFEWTPGYWQPYALPL; SWTDYGYWQPYALPISGL; ETPFTWEESNAYYWQPYALPL; ENTYSPNWADSMYWQPYALPL; SVGEDHNFWTSEYWQPYALPL; and DGYDRWRQSGERYWQPYALPL.

The present invention also provides conjugates of these peptides and derivatives and peptidomimetics of the peptides that retain the property of IL-1RtI binding but, by virtue of the conjugated compound, act either as an agonist or antagonist of IL-1RtI or direct a cytotoxic or other therapeutic agent to cells that express IL-1RtI.

The compounds described herein are useful for the prevention and treatment of diseases involving improper production of or response to IL-1 utilizing the novel compounds of the invention. Thus, the present invention also provides a method of treatment, wherein a patient having a disorder that is susceptible to treatment with an IL-1

inhibitor receives, or is administered, a therapeutically effective dose or amount of a compound of the present invention.

The invention also provides for pharmaceutical compositions comprising one or more of the compounds described herein and a physiologically acceptable carrier. These pharmaceutical compositions can be in a variety of forms including oral dosage forms, as well as injectable and infusible solutions. The present invention also provides conjugates of these peptides and derivatives and peptidomimetics of the peptides that retain the property of IL-1RtI binding but, by virtue of the conjugated compound, act either as an agonist or antagonist of IL-1RtI or direct a cytotoxic or other therapeutic agent to cells that express IL-1RtI.

DESCRIPTION OF THE PREFERRED EMBODIMENT

I. Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

"Pharmaceutically acceptable salts" refer to the non-toxic alkali metal, alkaline earth metal, and ammonium salts commonly used in the pharmaceutical industry including the sodium, potassium, lithium, calcium, magnesium, barium, ammonium, and protamine zinc salts, which are prepared by methods well known in the art. The term also includes non-toxic acid addition salts, which are generally prepared by reacting the compounds of this invention with a suitable organic or inorganic acid. Representative salts include the hydrochloride, hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napsylate, and the like.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases and which are not biologically or otherwise undesirable, formed with

inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, menthanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. For a description of pharmaceutically acceptable acid addition salts as prodrugs, *see* Bundgaard, H., *supra*.

"Pharmaceutically acceptable ester" refers to those esters which retain, upon hydrolysis of the ester bond, the biological effectiveness and properties of the carboxylic acid or alcohol and are not biologically or otherwise undesirable. For a description of pharmaceutically acceptable esters as prodrugs, *see* Bundgaard, H., ed., (1985) *Design of Prodrugs*, Elsevier Science Publishers, Amsterdam. These esters are typically formed from the corresponding carboxylic acid and an alcohol. Generally, ester formation can be accomplished via conventional synthetic techniques. (*See, e.g.,* March *Advanced Organic Chemistry*, 3rd Ed., John Wiley & Sons, New York (1985) p. 1157 and references cited therein, and Mark *et al. Encyclopedia of Chemical Technology*, John Wiley & Sons, New York (1980).) The alcohol component of the ester will generally comprise (i) a C_2-C_{12} aliphatic alcohol that can or can not contain one or more double bonds and can or can not contain branched carbon chains or (ii) a C_7-C_{12} aromatic or heteroaromatic alcohols. This invention also contemplates the use of those compositions which are both esters as described herein and at the same time are the pharmaceutically acceptable acid addition salts thereof.

"Pharmaceutically acceptable amide" refers to those amides which retain, upon hydrolysis of the amide bond, the biological effectiveness and properties of the carboxylic acid or amine and are not biologically or otherwise undesirable. For a description of pharmaceutically acceptable amides as prodrugs, *see* Bundgaard, H., ed., (1985) *Design of Prodrugs*, Elsevier Science Publishers, Amsterdam. These amides are

typically formed from the corresponding carboxylic acid and an amine. Generally, amide formation can be accomplished via conventional synthetic techniques. (*See, e.g.,* March *Advanced Organic Chemistry*, 3rd Ed., John Wiley & Sons, New York (1985) p. 1152 and Mark *et al. Encyclopedia of Chemical Technology*, John Wiley & Sons, New York (1980).) This invention also contemplates the use of those compositions which are both amides as described herein and at the same time are the pharmaceutically acceptable acid addition salts thereof.

"Pharmaceutically or therapeutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the host or patient.

"Stereoisomer" refers to a chemical compound having the same molecular weight, chemical composition, and constitution as another, but with the atoms grouped differently. That is, certain identical chemical moieties are at different orientations in space and, therefore, when pure, has the ability to rotate the plane of polarized light. However, some pure stereoisomers may have an optical rotation that is so slight that it is undetectable with present instrumentation. The compounds of the instant invention may have one or more asymmetrical carbon atoms and therefore include various stereoisomers. All stereoisomers are included within the scope of the invention.

"Therapeutically- or pharmaceutically-effective amount" as applied to the compositions of the instant invention refers to the amount of composition sufficient to induce a desired biological result. That result can be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. In the present invention, the result will typically involve a decrease in the immunological and/or inflammatory responses to infection or tissue injury.

Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L;

Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

In addition to peptides consisting only of naturally-occurring amino acids, peptidomimetics or peptide analogs are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) *TINS* p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as naturally-occurring receptor-binding polypeptide, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: $-CH_2NH-$, $-CH_2S-$, $-CH_2-CH_2-$, -CH=CH- (*cis* and *trans*), $-COCH_2-$, $-CH(OH)CH_2-$, and $-CH_2SO-$, by methods known in the art and further described in the following references: Spatola, A.F. in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, *Peptide Backbone Modifications* (general review); Morley, *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D. *et al.*, (1979) *Int J Pept Prot Res* 14:177-185 ($-CH_2NH-$, CH_2CH_2-); Spatola *et al.*, (1986) *Life Sci* 38:1243-1249 ($-CH_2-S$); Hann (1982) *J. Chem. Soc.* Perkin Trans. I 307-314 (-CH-CH-, *cis* and *trans*); Almquist *et al.*, (1980) *J Med Chem* 23:1392-1398 ($-COCH_2-$); Jennings-White *et al.*, (1982) *Tetrahedron Lett* 23:2533

(-$COCH_2$-); Szelke *et al.*, (1982) European Appln. EP 45665 CA: 97:39405 (1982) (-$CH(OH)CH_2$-); Holladay *et al.*, (1983) *Tetrahedron Lett* 24:4401-4404 (-$C(OH)CH_2$-); and Hruby (1982) *Life Sci* 31:189-199 (-CH_2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH_2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (*e.g.*, immunoglobulin superfamily molecules) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic. Generally, peptidomimetics of receptor-binding peptides bind to the receptor with high affinity and possess detectable biological activity (i.e., are agonistic or antagonistic to one or more receptor-mediated phenotypic changes).

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch (1992) *Ann. Rev. Biochem.* 61: 387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide. Such D-amino acids are abbreviated by the lower case single letter

abbreviations previously described herein, *e.g.*, where "A" represents L-alanine and "a" represents D-alanine.

II. Overview

The present invention provides compounds that bind to the IL-1RtI. These compounds include "lead" peptide compounds and "derivative" compounds constructed so as to have the same or similar molecular structure or shape as the lead compounds but that differ from the lead compounds either with respect to susceptibility to hydrolysis or proteolysis and/or with respect to other biological properties, such as increased affinity for the receptor. The present invention also provides compositions comprising an effective IL-1RtI binding, IL-1 blocking compound, and more particularly a compound, that is useful for treating disorders associated with the overexpression of IL-1.

In general, the peptides of the present invention will have strong binding properties to the Interleukin-1 type I receptor ("IL-1RtI"). Specifically, the peptides will be characterized as having a binding affinity to IL-1RtI as measured by an IC_{50} of about 2.5 mM or less, typically, about 100 μM or less, and preferably, 20 μM or less. Additionally, the peptides of the present invention may be relatively small. Typically, the peptides have a molecular weight of less than about 5000 kilodaltons, and preferably, less than about 3000 kilodaltons.

III. Random Peptide Diversity Generating Systems

Initial lead peptide compounds were identified using random peptide diversity generating systems including the "peptides on phage" and "peptides on plasmids" systems discussed above and described in U.S. Patent No. 5,270,170, and co-pending U.S. application Serial Nos. 718,577, and 07/847,567, filed March 5, 1992. The random peptides were designed to be eight to twelve amino acid residues in length, and one system employed fixed cysteine residues at each end of the random peptide to facilitate the formation of cyclic peptides. To generate the collection of oligonucleotides that

encode the random peptides, the codon motif (NNK)x, where N was nucleotide A, C, G, or T (equimolar; depending on the methodology employed, other nucleotides can be employed), K is G or T (equimolar), and x was 6 (for the cyclic library--the other two terminal codons were cysteine codons), 8, 10, or 12; was used in the synthesis of the oligonucleotides. Those of skill in the art will recognize that the NNK motif encodes all of the amino acids, encodes only one stop codon, and reduces codon bias. There are 32 possible codons resulting from the NNK motif: 1 for each of 12 amino acids, 2 for each of 5 amino acids, 3 for each of 3 amino acids, and only one of the three stop codons.

In these initial systems, the random peptides were presented as part of a fusion protein comprising either the pIII or pVIII coat protein of a phage fd derivative (peptides on phage) or the lac repressor DNA binding protein (peptides on plasmids). The fusion proteins, along with the DNA encoding the fusion proteins, were "panned" on immobilized IL-1RtI. The panning process involved multiple rounds of incubating the fusion proteins with the immobilized receptor, collecting the fusion proteins that bound to the receptor (along with the accompanying DNA), and amplifying the fusion proteins collected.

Typically after three rounds of panning, the fusion proteins and accompanying DNA were isolated and cultured to produce fusion protein preparations for an ELISA to determine if the fusion protein bound specifically to the receptor. This assay was carried out similarly to the panning, except that after removing unbound fusion proteins, the wells were treated with rabbit anti-phage antibody (or with anti-lac antibody for the peptides on plasmids system), then with alkaline phosphatase-conjugated goat anti-rabbit antibody, and then the amount of alkaline phosphatase in each well was determined by standard methods. By comparing test wells with control wells (no receptor), one can determine whether the fusion proteins bind to the receptor specifically. Fusion proteins found to bind specifically to the receptor were then tested in an IL-1α blocking assay. The blocking assay was

carried out in similar fashion to the ELISA , except that IL-1α was added to the wells before the fusion protein (the control wells were of two types: (1) no receptor; and (2) no IL-1α). Fusion proteins for which the binding to the receptor was blocked by IL-1α contain peptides in the random peptide portion that are preferred compounds of the invention.

Several forms of the Type I IL-1 receptor were used in the panning and ELISA procedures and IC_{50} determinations. For example, radiolabelled monovalent receptor was prepared by expressing soluble receptor fused with the 7 amino acid substrate sequence for protein kinase A. The receptor was then labelled using protein kinase A and ^{33}P or ^{32}P-ATP. See, Li, B. L., et al., Creation of phosphorylation sites in proteins: construction of a phosphorylatable human interferon alpha *Proc. Natl Acad. Sci.* 86(2):558-562. Additionally, an immobilized receptor useful in the panning and ELISA procedures was produced in recombinant host cells in a truncated form comprising the complete extracellular domain (as determined by hydrophobicity studies) of IL-1RtI (amino acids 1 to 336 of the sequence reported by Chua and Gubler, 1989, *Nuc. Acids Res.* 17(23):10114, incorporated herein by reference). This truncated receptor molecule can be produced in a variety of different forms and host cells. One useful form of the receptor is constructed by expressing the receptor as a soluble protein in baculovirus transformed host cells using standard methods; another useful form is constructed with a signal peptide for protein secretion and for glycophospholipid membrane anchor attachment (this form of anchor attachment is called "PIG-tailing;" *see* Caras and Weddell (1989) *Science* 243:1196-1198, and Lin *et al.*, (1990) *Science* 249:677-679, each of which is incorporated herein by reference). Using the latter system, one can cleave the receptor from the surface of the cells expressing the receptor and collect the cleaved receptor quite easily.

Several bivalent forms of the receptor were also prepared for use in the identification of peptides capable of interacting with the Il-1 receptor. A bivalent receptor-antibody complex was generated by mixing ^{33}P labeled

receptor with an anti-IL-1RtI antibody followed by the addition of excess unlabeled receptor. Similarly, a bivalent receptor was obtained by mixing an excess of the receptor with ^{125}I labeled antibody, and then purifying the receptor by passing it over an Ab179 column (if one is using the PIG-tailed form of the receptor, then one can use an antibody that binds to the HPAP residue that remains attached to the receptor after secretion and cleavage). Another bivalent receptor was constructed by linking the extracellular portion of the Type I IL-1R through its C-terminus to the Fc portion of a human IgG molecule.

The recombinant receptor protein was immobilized using the following methodology. Microtiter plates were coated with an anti-IL-1RtI antibody that does not block IL-1α binding to IL-1RtI and then the wells containing the immobilized receptor were treated with bovine serum albumin (BSA) to block non-specific binding. The receptor was added to the coated wells of the microtiter plate, which were then washed to remove unbound receptor.

Often, the receptor was added only to alternate rows of the microtiter plate; the BSA-blocked wells in the "blank" rows served as useful negative controls to determine whether a receptor-specific reaction was creating the observed results. Fusion protein preparations were then added to the wells and incubated to allow binding to the receptor to occur; then, the wells were washed to remove unbound fusion proteins.

With the above systems, a number of different fusion proteins were discovered that bind to the IL-1RtI, but that binding did not appear to be blocked by IL-1α at a concentration of about 12 μM. The DNA encoding the fusion proteins that bound to the receptor was sequenced to determine the sequence of the random peptide that the fusion proteins contained. These peptides, together with similar peptides isolated using different libraries (discussed below) are shown in Table 1, below.

Table 1

Fusion Protein	Random Peptide Sequence
R1	WWTDTGLW
R11	WWTDDGLW
S4	WWDTRGLWVWTI
DB29	FWGNDGIWLESG
S14	DWDQFGLWRGAA
NU1	RWDDNGLWVVVL
T11	SGMWSHYGIWMG
T12	GGRWDQAGLWVA
MC1	KLWSEQGIWMGE
CYC1	CWSMHGLWLC
F17	GCWDNTGIWVPC
IL1	DWDTRGLWVY
IL3	SLWDENGAWI
IL4	KWDDRGLWMH
IL6	QAWNERGLWT
IL7	QWDTRGLWVA
IL8,11E	WNVHGIWQE
IL9	SWDTRGLWVE
IL12,17	DWDTRGLWVA
IL13	SWGRDGLWIE
IL16	EWTDNGLWAL
IL19	SWDEKGLWSA
IL20	SWDSSGLWMD

The peptides in Table 1 are characterized by the motif "WXXXGZ$_1$W" where each amino acid is indicated by standard one letter abbreviation; each X can be selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; and Z_1 is L, I, A, or Q. More preferably, the core sequence of amino acids will be comprise WZ_2XXGZ_1W where X can be selected from any one of the 20 genetically coded L-amino acids; Z_1 is L, I, A, or Q; and Z_2 is D, G, N, S, or T. In a more preferred embodiment, the core sequence of amino acids will comprise $WZ_2Z_3Z_4GZ_1W$ where Z_1 is L, I, A, or Q; Z_2 is D, G, N, S, or T; Z_3, is D, E, H, M, N, Q, R, S, T, or V; and Z_4 is A, D, F, H, K, N, Q, R, T, or Y. Most preferably, the core sequence of amino acids comprise

$WZ_2Z_3Z_4GZ_1W$ where Z_1 is L or I; Z_2 is D, S, or T; Z_3 is D, E, or T; and Z_4 is D, H, N, R, or T. An especially preferred embodiment is one having the substitution patterns just described, but comprising 8, 10, or 12 amino acid residues. An especially preferred peptide has the sequence SWDTRGLWVE. These peptides are valuable due to their ability to bind specifically with the IL-1R without blocking the IL-1α binding site on the receptor.

The peptides of the present invention can be conjugated to compounds that do bind to the IL-1α binding site of an IL-1R to construct compounds with an affinity for the IL-1R greater than either of the compounds of which the conjugate is composed. The discovery of these peptides also facilitates the identification of peptides that do bind to the same site on IL-1R as IL-1α, because one can bias the library or panning procedure to eliminate peptides with this "non-blocking" motif. For instance, one can make a library with no tryptophan residues in the random peptide or one can pan a library using receptor immobilized with an anti-IL-IR antibody that blocks binding of peptides with this motif but does not block IL-1 binding. One could also pan in the presence of high concentrations of the peptide.

Several new libraries of random peptides were constructed in a manner designed to minimize the production of fusion proteins comprising the "$WXXXGZ_1W$" motif. These libraries were characterized by the use of the codon motif "NNW," where W is nucleotide A or T, in codons two and eight in synthesizing the oligonucleotide encoding the random peptide. The resulting peptides thus did not contain a methionine (M) or a tryptophan (W) at positions two and eight of the random peptide portion of the fusion protein. Panning of these libraries yielded fusion proteins T11, T12, and F17 in Table 1, above, together with the fusion protein T6, which comprises the peptide "RLVYWQPYSVQR". Phage bearing the fusion protein T6 bound specifically to IL-1RtI, and binding of the phage was inhibited by IL-1α. The T6 peptide was synthesized on a peptide synthesizer (Example 1, below), and the synthetic free peptide was tested and found to compete

with IL-1α in binding to the IL-1R at micromolar concentration (*see* Example 3).

The T6 peptide sequence then served as the basis for the construction of another peptide library designed to contain a high frequency of derivatives of the T6 peptide. This library was synthesized so as to favor the production of peptides that differ from the T6 peptide in only a few residues. This approach involved the synthesis of an oligonucleotide with the T6 random peptide coding sequence, except that rather than using pure preparations of each of the four nucleoside triphosphates in the synthesis, mixtures of the four nucleoside triphosphates were used so as to generate derivatives of the T6 peptide coding sequence. This library was prepared and panned as above, and the peptide sequences obtained are shown in Table 2, below, together with the T6 sequence.

IC_{50} values are indicated symbolically by the symbols "-", "+", and "++". For example, those peptides which showed IC_{50} values in excess of 100 μM are indicated with a "-". Those peptides which give IC_{50} values of less than or equal to 100 μM are given a "+", while those which give IC_{50} values of 500 nM or less are indicated with a "++". Those peptides which give IC_{50} values at or near the cutoff point for a particular symbol are indicated with a hybrid, e.g. "+/-". Those peptides for which IC_{50} values were not determined are listed as "N.D".

Table 2

Fusion Protein	Random Peptide Sequence	IC_{50}[1]
2H2	SHLYWQPYSVQ	+
H3	TLVYWQPYSLQT	+
3H9, 11	RGDYWQPYSVQS	+
3H5	VHVYWQPYSVQT	+
3H2	RLVYWQPYSVQT	+
3H1, 6, 12	SRVWFQPYSLQS	+

[1] Unless otherwise indicated, IC_{50} determinations were conducted using soluble peptide. Results marked with * were obtained using the immobilized PIG-tailed Type I IL-1R.

3H7	NMVYWQPYSIQT	+
3H10	SVVFWQPYSVQT	+
3H4	TFVYWQPYALPL	+
2H11	TLVYWQPYSIQR	+
T6	RLVYWQPYSVQR	+
D1	SPVFWQPYSIQI	+
D2	WIEWWQPYSVQS	+
D3	SLIYWQPYSLQM	+
D13	TRLYWQPYSVQR	+
D15	RCDYWQPYSVQT	+
D18	MRVFWQPYSVQN	+
D19	KIVYWQPYSVQT	+
D20	RHLYWQPYSVQR	+
2H6	ALVWWQPYSEQI	+
3H8	SRVWFQPYSLQS	

Table 2 shows that a general structure for these compounds is defined by $Z_7XQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze (or J) where Aze is azetidine; Z_6 is S, A V, or L; and Z_7 is Y, W, or F.

In addition, screening of a 10-mer library on PVIII in the presence of a known blocking peptide, SWDTRGLWVE, resulted in the identification of the following peptides:

Table 3

Peptide	IC_{50}
WEQPYALPLE	+
REYEQPYALW	-
EEWAQPYAFL	-
GSWEQPYAMV	-
AWYGPSNLPV	-

The results of screening of various other libraries (for example, 70:10:10:10; fixed-sliding libraries and extended/mutagenized libraries which were screened using standard elution conditions and tested under standard or low receptor density conditions) are shown below:

Table 4

Library Based on RLVYWQPYSVQR with 50% NNK

Peptide	IC_{50}
QLVWWQPYSVQR	+
DLRYWQPYSVQV	+
ELVWWQPYSLQL	+
DLVWWQPYSVQW	+
NGNYWQPYSFQV	+
ELVYWQPYSIQR	+
WSNYWQPYSVQP	+/-
QYVYWQPLSVQV	-
ELMYWQPYSVQE	N.D.
NLLYWQPYSMQD	N.D.

Table 5

Library Based on XXXXXXPYSVQR

Peptide	IC_{50}
GYEWYQPYSVQR	+
SRVWYQPYSVQR	+
LSEQYQPYSVQR	+
GGGWWQPYSVQR	+
VGRWYQPYSVQR	+
VHVYWQPYSVQR	+
QARWYQPYSVQR	+
VHVYWQPYSVQT	+
RSVYWQPYSVQR	+
TRVWFQPYSVQR	+
GRIWFQPYSVQR	+
GRVWFQPYSVQR	+
ARTWYQPYSVQR	+
GRLWWQPYSVQR	+/-
HRIWWQPYSVQR	+/-
GRVWWQPYSVQR	+/-
ARVWWQPYSVQM	N.D.
RLMFYQPYSVQR	N.D.
ESMWYQPYSVQR	N.D.
HFGWWQPYSVHM	N.D.
ARFWWQPYSVQR	N.D.

Table 6

Library Based on "RLVYWQ XXXXXX

Peptide	IC_{50}
RLVYWQ PYAPIY	+
RLVYWQ PYSYQT	+
RLVYWQ PYSLPI	+
RLVYWQ PYSVQA	+
RLVYWQ PWAPIW	-

Table 7

Library Based on "SRVWYQ XXXXXX"

Peptide	IC_{50}
SRVWYQ PYAKGL	+
SRVWYQ PYAQGL	+
SRVWYQ PYAMPL	+
SRVWYQ PYSVQA	+
SRVWYQ PYSLGL	+
SRVWYQ PYAREL	+
SRVWYQ PYSRQP	+
SRVWYQ PYFVQP	+/-

Table 8

Library Based on "XXXXXXPYALPL"

Peptide	IC_{50}
EYEWYQ PYALPL	+
IPEYWQ PYALPL	+
SRIWWQ PYALPL	+
DPLFWQ PYALPL	+
SRQWVQ PYALPL	+
IRSWWQ PYALPL	+
RGYWQ PYALPL	+
RLLWVQ PYALPL	+
EYRWFQ PYALPL	+
DAYWVQ PYALPL	+
WSGYFQ PYALPL	+
NIEFWQ PYALPL	+
TRDWVQ PYALPL	+
DSSWYQ PYALPL	+

IGNWYQ PYALPL	+
NLRWDQ PYALPL	+
LPEFWQ PYALPL	+
DSYWWQ PYALPL	+
RSQYYQ PYALPL	+
ARFWLQ PYALPL	+
NSYFWQ PYALPL	+
ESFWVQ PYALPL	-

Table 9

Library Based on "RLVYWQPYSVQR" with 70-10-10-10 Mutagenesis

Peptide	IC_{50}
RFMYWQPYSVQR	+
AHLFWQPYSVQR	+
WGNWWQPYSVHR	-

Table 10

Library Based on "XXQPYXXXX"

Peptide	IC_{50}
WWQPYALPL	+
YYQPYALPL	+
YFQPYALGL	+
YFQPYALPF	+

Table 11

Library Based on "XXXQPYXXXX"

Peptide	IC_{50}
YWYQPYALPL	+
RWWQPYATPL	+
GWYQPYALGF	+
YWYQPYALGL	+
IWYQPYAMPL	+
SNMQPYQRLS	N.D.

Table 12

Library Based on "TFVYWQPY XXXXXXXXXXXX"

Peptide
TFVYWQPY AVGLFAAETACN
TFVYWQPY SVQMTITGKVTM
TFVYWQPY SSHXXVPXGFPL
TFVYWQPY YGNPQWAIHVRH
TFVYWQPY VLLELPEGAVRA
TFVYWQPY VDYVWPIPIAQV

A library based on the following motif "XXXXXX(PG/GP)XXXXXX" where X represents any of the naturally occurring L-amino acids was also prepared. Screening of this library resulted in the identification of the peptide IMWFCQPGGACYSV which had an IC_{50} greater than 100 μM.

The peptides-on-plasmid system was also employed to identify peptides capable of interacting with the IL-1 receptor. Using this technique, a plasmid containing the LacI gene with an oligonucleotide cloning site at its 3'-end was constructed. Under the controlled induction by arabinose, a LacI-peptide fusion protein was produced. This fusion retains the natural ability of LacI to bind to the Lac operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each cell expresses only a single peptide sequence, the peptides become specifically associated with the DNA sequence that directed its synthesis. The cells of the library were gently lysed and the peptide-DNA complexes were exposed to a matrix of immobilized receptor to recover the complexes containing the active peptides. The associated plasmid DNA was then reintroduced into cells for amplification and DNA sequencing to determine the identity of the peptide ligands. Using this system, the following peptides capable of interacting with the IL-1 receptor were identified.

Table 13

Library Based on "XXXQPYXXXXXX" using C-terminal lac Repressor System

Peptide	IC_{50}
GWYQPYVDGWR	+
RWEQPYVKDGWS	+
EWYQPYALGWAR	+
GWWQPYARGL	+
LFEQPYAKALGL	+
GWEQPYARGLAG	+
AWVQPYATPLDE	+
MWYQPYSSQPAE	+
GWTQPYSQQGEV	+
DWFQPYSIQSDE	+
PWIQPYARGFG	+

A library was also screened by panning against IL-1 receptor expressed by cells to identify the following peptides:

Table 14

Peptide	IC_{50}
RPLYWQPYSVQV	+
TLIYWQPYSVQI	+
RFDYWQPYSDQT	+
WHQFVQPYALPL	N.D.

When using random peptide generation systems that allow for multivalent ligand-receptor interaction, one must recognize that the density of the immobilized receptor is an important factor in determining the affinity of the ligands that bind to the immobilized receptor. At higher receptor densities (i.e., each anti-receptor antibody-coated well treated with 250 to 500 ng of receptor), multivalent binding is more likely to occur (if at all) than at lower receptor densities (i.e., each anti-receptor antibody-coated well treated with 0.5 to 1 ng of the receptor). If multivalent binding is occurring, then one will be more likely to isolate ligands with relatively low affinity. Typically, one can

identify lead compounds using a high density of immobilized receptor and then test the derivatives of the lead compound at lower receptor densities to isolate compounds with higher affinity for the receptor than the lead compound. By screening libraries under conditions of low receptor density, the following peptides were identified.

Table 15

Library Based on "XXXX VYWQPYSVQ XXXX" with Low Density Receptor

Peptide
EWDS VYWQPYSVQ TLLR
WEQN VYWQPYSVQ SFAD
SDV VYWQPYSVQ SLEM
YYDG VYWQPYSVQ VMPA

Table 16

Library Based on "XXXXXX PYALPL" with Low Density Receptor

Peptide	IC_{50}
SDIWYQ PYALPL	+
QRIWWQ PYALPL	+
SRIWWQ PYALPL	+
RSLYWQ PYALPL	+
TIIWEQ PYALPL	+
WETWYQ PYALPL	+
SYDWEQ PYALPL	+
SRIWCQ PYALPL	+
EIMFWQ PYALPL	+
DYVWQQ PYALPL	+

Table 17

Library Based on "XXXXXX WYQPYALPL" with Low Density Receptor

Peptide
MDLLVQ WYQPYALPL
GSKVIL WYQPYALPL
RQGANI WYQPYALPL

GGGDEP WYQPYALPL
SQLERT WYQPYALPL
ETWVRE WYQPYALPL
KKGSTQ WYQPYALPL
LQARMN WYQPYALPL
EPRSQK WYQPYALPL
VKQKWR WYQPYALPL
LRRHDV WYQPYALPL
RSTASI WYQPYALPL
ESKEDQ WYQPYALPL
EGLTMK WYQPYALPL
EGSREG WYQPYALPL

A "monovalent" phage approach was also used to identify peptides capable of binding the IL-1 receptor. In this approach, phage particles with only a single chimeric pIII protein were created, thereby eliminating multivalent binding to immobilized receptor. Using this technique, the following peptides were identified.

Table 18

Library Based on "XXXXXX PYALPL" with Monovalent Display

Peptide	IC_{50}
VIEWWQ PYALPL	+
VWYWEQ PYALPL	+
ASEWWQ PYALPL	+
FYEWWQ PYALPL	+
EGWWVQ PYALPL	+
WGEWLQ PYALPL	+
DYVWEQ PYALPL	+
AHTWWQ PYALPL	+
FIEWFQ PYALPL	+
WLAWEQ PYALPL	+
VMEWWQ PYALPL	N.D.
ERMWQ PYALPL	N.D.

To ascertain a rough indication of the affinity of the peptides, selected phage libraries were also screened

using an affinity selection protocol. In brief, this protocol relies on the rapid association and dissociation between the peptide on an individual phage (either pIII or pVIII) and the receptor. More specifically, for phage bearing low affinity ligands, the peptide on an individual pIII (or pVIII) protein may be rapidly dissociating and reassociating, but the phage particle will not dissociate unless all the peptides on the phage are simultaneously in the unbound state. Dissociation of the phage can be initiated by addition of a competing peptide, which prevents rebinding of any individual peptide in the complex. The concentration (and affinity) of the competing peptide, as well as the time and temperature of elution can be varied to select for peptides of various affinities.

Thus, to identify peptides capable of interacting with the IL-1 receptor, competition with a peptide or IL-1 was performed. This process is repeated typically for two rounds of panning. In subsequent rounds of panning, the competition temperature (4°C to ambient temperature) and time (15 to 30 minutes) as well as the temperature (4°C to ambient temperature) of the wash solutions can be altered to further select for peptides with high affinity.

Table 19

Library Based on "XXXXXX PYALPL" with Peptide Competition

Peptide	IC_{50}
NXXWXX PYALPL	N.D.
WGNWYQ PYALPL	N.D.
TLYWEQ PYALPL	N.D.
VWRWEQ PYALPL	N.D.
LLWTQ PYALPL	N.D.
SRIWXX PYALPL	N.D.
SDIWYQ PYALPL	N.D.
WGYYXX PYALPL	N.D.
TSGWYQ PYALPL	N.D.
VHPYXX PYALPL	N.D.
EHSYFQ PYALPL	N.D.

XXIWYQ PYALPL	N.D.
AQLHSQ PYALPL	N.D.
WANWFQ PYALPL	N.D.
SRLYSQ PYALPL	N.D.
YYTWQQ PYALPL	-
GVTFSQ PYALPL	N.D.
GVVWYQ PYALPL	-
SIVWSQ PYALPL	N.D.
YYSWQ PYALPL	-
SRDLVQ PYALPL	N.D.

Table 20

Library Based on "XXXX VYWQPYSVQ XXXX"

with IL-1 Competition

Peptide	IC_{50}
HWGH VYWQPYSVQ DDLG	+
SWHS VYWQPYSVQ SVPE	+
WRDS VYWQPYSVQ PESA	+
TWDA VYWQPYSVQ KWLD	N.D.
TPPW VYWQPYSVQ SLDP	N.D.
YWSS VYWQPYSVQ SVHS	N.D.

Table 21

Peptides Identified from Library Based on

"XXX QPY XXXX" with Peptide Competition

Peptide
YWY QPY ALGL
YWY QPY ALPL
EWI QPY ATGL
NWE QPY AKPL
AFY QPY ALPL
FLY QPY ALPL
VCK QPY LEWC

To more clearly define the preferred sequences, several additional libraries were screened using a colony lift technique. In brief, cells were infected with phage encoding random peptides and were plated on media containing arabinose

to induce expression of the random peptides. Colonies were transferred to nitrocellulose filters which were washed extensively and then incubated at 4°C with ^{33}P radiolabeled IL-1R. The filters were washed, dried, and exposed to X-ray film. The sequences of peptides identified using this technique are shown below.

Table 22

Peptides Identified by Colony Lifts from Library Based on "XXXXXXXXXXXXX YWQPYALPL"

Peptide	IC_{50}
SVGEDHNFWTSE YWQPYALPL	+/++
MNDQTSEVSTFP YWQPYALPL	N.D.
SWSEAFEQPRNL YWQPYALPL	N.D.
QYAEPSALNDWG YWQPYALPL	N.D.
NGDWATADWSNY YWQPYALPL	N.D.
THDEHI YWQPYALPL	N.D.
MLEKTYTTWTPG YWQPYALPL	N.D.
WSDPLTRDADL YWQPYALPL	N.D.
SDAFTTQDSQAM YWQPYALPL	N.D.
GDDAAWRTDSLT YWQPYALPL	N.D.
AIIRQLYRWSEM YWQPYALPL	N.D.
ENTYSPNWADSM YWQPYALPL	++
ETPFTWEESNAY YWQPYALPL	++
MNDQTSEVSTFP YWQPYALPL	N.D.
SVGEDHNFWTSE YWQPYALPL	N.D.
DGYDRWRQSGER YWQPYALPL	++
TANVSSFEWTPG YWQPYALPL	++
QTPFTWEESNAY YWQPYALPL	N.D.
ENPFTWQESNAY YWQPYALPL	N.D.
VTPFTWEDSNVF YWQPYALPL	N.D.
QIPFTWEQSNAY YWQPYALPL	N.D.
QAPLTWQESAAY YWQPYALPL	N.D.
EPTFTWEESKAT YWQPYALPL	N.D.
TTTLTWEESNAY YWQPYALPL	N.D.
ESPLTWEESSAL YWQPYALPL	N.D.
ETPLTWEESNAY YWQPYALPL	N.D.

EATFTWAESNAY YWQPYALPL	N.D.
EALFTWKESTAY YWQPYALPL	N.D.
STP-TWEESNAY YWQPYALPL	N.D.
ETPFTWEESNAY YWQPYALPL	N.D.
KAPFTWEESQAY YWQPYALPL	N.D.
STSFTWEESNAY YWQPYALPL	N.D.
DSTFTWEESNAY YWQPYALPL	N.D.
YIPFTWEESNAY YWQPYALPL	N.D.
QTAFTWEESNAY YWQPYALPL	N.D.
ETLFTWEESNAT YWQPYALPL	N.D.
VSSFTWEESNAY YWQPYALPL	N.D.

The peptide ETPFTWEESNAYYWQPYALPL was truncated at the N terminus, and the proline in the QPY motif was substituted with azetidine to yield the peptide FTWEESNAYYWQJYALPL, which similarly had an IC_{50} of less than 500 nM.

Additionally, truncation of the peptide TANVSSFEWTPG YWQPYALPL yielded the peptide FEWTPGYWQPYALPL having an IC_{50} of less than 500 nM. Substitution of the proline in the QPY motif with azetidine yielded a peptide of similar affinity, as did substitution of the tryptophan residue within the YW motif with tyrosine. These peptides are thus FEWTPGYWQJYALPL and FEWTPGYYQJYALPL.

Table 23

Other Peptides Identified by Colony Lifts with Labeled Receptor

Peptide	IC_{50}
ADVL YWQPYA PVTLWV	N.D.
GDVAE YWQPYA LPLTSL	N.D
SWTDYG YWQPYA LPISGL	++

The above data illustrate a peptide comprising a core sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; and Z_6 is S, A,

V, or L. More preferably, the sequence comprises $Z_7XQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; Z_6 is S or A; and Z_7 is Y, W, or F. In a more preferred embodiment, the core sequence of amino acids will comprise $Z_7Z_8QZ_5YZ_6Z_9Z_{10}$ where Z_5 is P or Aze where Aze is azetidine; Z_6 is S or A; Z_7 is Y, W or F; Z_8 is E, F, V, W, or Y; Z_9 is M, F, V, R, Q, K, T,S, D, L, I, or E; and Z_{10} is E, L, W, V, H, I, G, A, D, L, Y, N, Q or P. More preferably, Z_9 is V, L, I, or E; and Z_{10} is Q or P. Most preferably, the core peptide will comprise a sequence of amino acids $Z_{11}Z_7Z_8QZ_5YZ_6Z_9Z_{10}$, where Z_8 is Y, W or F; and Z_{11} is V, L, I, E, P, G, Y, M, T, or, D. Specific examples of these peptides include FEWTPGYWQPYALPL, FEWTPGYWQJYALPL, FEWTPGWYQPYALPL, FEWTPGWYQJYALPL, FEWTPGYYQPYALPL and FEWTPGYYQJYALPL.

An especially preferred embodiment is one having the substitution patterns just described, but comprising 21 amino acid residues. Particularly preferred is the peptide comprising the sequence of amino acids $Z_{12}Z_{13}Z_{14}Z_{15}Z_{16}Z_{17}Z_{18}Z_{19}Z_{20}Z_{21}Z_{22}Z_{11}Z_7Z_8QZ_5YZ_6Z_9Z_{10}L$, where Z_8 is Y, W or F; Z_{11} is V, L, I, E, P, G, Y, M, T, or, D; Z_{12} is A, D, E, F, G, K, Q, S, T, V, or Y; Z_{13} is A, D, G, I, N, P, S, T, V, or W; Z_{14} is A, D, G, L, N, P, S, T, W, or Y; Z_{15} is A, D, E, F, L, N, R, V, or Y; Z_{16} is A, D, E, Q, R, S, or T; Z_{17} is H, I, L, P, S, T, or, W; Z_{18} is A, E, F, K, N, Q, R, S, or Y; Z_{19} is D, E, F, Q, R, T, or W; Z_{20} is A, D, P, S, T, or W; Z_{21} is A, D, G, K, N, Q, S, or T; and Z_{22} is A, E, L, P, S, T, V, or Y. More preferably, Z_8 is Y, W or F; Z_{11} is V, L, I, E, P, G, Y, M, T, or, D; Z_{12} is D, E, Q, S, T, V, or Y; Z_{13} is A, D, G, I, N, S, T or V; Z_{14} is A, G, L, N, P, S, T, or Y; Z_{15} is D, E, F, L, V, or Y; Z_{16} is D, R, S or T; Z_{17} is H, P, S, or W; Z_{18} is E, F, N, R, Q, or S; Z_{19} is D, E, F, Q, or W; Z_{20} is S, T, or W; Z_{21} is D, G, K, N, Q, S, or T; and Z_{22} is A, E, P, S, or Y.

Particularly preferred peptides include TANVSSFEWTPGYWQPYALPL; SWTDYGYWQPYALPISGL; ETPFTWEESNAYYWQPYALPL; ENTYSPNWADSMYWQPYALPL; SVGEDHNFWTSEYWQPYALPL; and DGYDRWRQSGERYWQPYALPL.

The preferred motif sequences also provide a means to determine the minimum size of an IL-1R blocking compound of the invention. Using the "encoded synthetic library" (ESL) system described in U.S. patent application Serial No. 946,239, filed September 16, 1992, which is a continuation-in-part application of Serial No. 762,522, filed September 18, 1991, or the "very large scale immobilized polymer synthesis" system described in U.S. patent application Serial Nos. 492,462, filed March 7, 1990; 624,120, filed December 6, 1990; and 805,727, filed December 6, 1991; one can not only determine the minimum size of a peptide with such activity, one can also make all of the peptides that form the group of peptides that differ from the preferred motif (or the minimum size of that motif) in one, two, or more residues. This collection of peptides can then be screened for ability to bind to IL-1-receptor. This immobilized polymer synthesis system or other peptide synthesis methods can also be used to synthesize every truncation analog and every deletion analog and every combination of truncation and deletion analog of all of the peptide compounds of the invention.

The peptides of the invention can be prepared by classical methods known in the art, for example, by using standard solid phase techniques. The standard methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis, and even by recombinant DNA technology. *See, e.g.*, Merrifield (1963) *J. Am. Chem. Soc.* 85:2149, incorporated herein by reference. On solid phase, the synthesis is typically commenced from the C-terminal end of the peptide using an alpha-amino protected resin. A suitable starting material can be prepared, for instance, by attaching the required alpha-amino acid to a chloromethylated resin, a hydroxymethyl resin, or a benzhydrylamine resin. One such chloromethylated resin is sold under the trade name BIO-BEADS SX-1 by Bio Rad Laboratories, Richmond, CA, and the preparation of the hydroxymethyl resin is described by Bodonszky *et al.*, (1966) *Chem. Ind. (London)* 38:1597. The benzhydrylamine (BHA) resin has been described by Pietta and Marshall (1970) *Chem. Commn.*

650, and is commercially available from Beckman Instruments, Inc., Palo Alto, CA, in the hydrochloride form.

Thus, the compounds of the invention can be prepared by coupling an alpha-amino protected amino acid to the chloromethylated resin with the aid of, for example, cesium bicarbonate catalyst, according to the method described by Gisin (1973) *Helv. Chim. Acta* 56:1467. After the initial coupling, the alpha-amino protecting group is removed by a choice of reagents including trifluoroacetic acid (TFA) or hydrochloric acid (HCl) solutions in organic solvents at room temperature.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups (*e.g.*, formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (*e.g.*, benzyloxycarboyl (Cbz) and substituted Cbz), aliphatic urethane protecting groups (*e.g.*, t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (*e.g.*, benzyl, triphenylmethyl). Boc and Fmoc are preferred protecting groups. The side chain protecting group remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

The side chain protecting groups for Tyr include tetrahydropyranyl, tert-butyl, trityl, benzyl, Cbz, Z-Br-Cbz, and 2,5-dichlorobenzyl. The side chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl, and cyclohexyl. The side chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl, and Cbz. The side chain protecting group for Thr and Ser is benzyl. The side chain protecting groups for Arg include nitro, Tosyl (Tos), Cbz, adamantyloxycarbonyl mesitoylsulfonyl (Mts), or Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. An excess of each protected amino acid is generally used with an appropriate carboxyl group activator such as dicyclohexylcarbodiimide (DCC) in solution, for example, in methylene chloride (CH_2Cl_2), dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treatment with a reagent such as trifluoroacetic acid or hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamide or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

In preparing the compounds of the invention, the resins used to prepare the peptide acids are employed, and the side chain protected peptide is cleaved with base and the appropriate alcohol, i.e., methanol. Side chain protecting groups are then removed in the usual fashion by treatment with hydrogen fluoride to obtain the desired ester. These solid phase peptide synthesis procedures are well known in the art and further described in Stewart, *Solid Phase Peptide Syntheses* (Freeman and Co., San Francisco, 1969).

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two, or more positions of any of the compounds of the invention. For instance, naphthylalanine can be substituted

for tryptophan, facilitating synthesis. Other synthetic amino acids that can be substituted into the peptides of the present invention include L-hydroxypropyl, L-3, 4-dihydroxyphenylalanyl, α amino acids such as L-α-hydroxylysyl and D-α-methylalanyl, L-α-methylalanyl, ß amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention.

One can replace the naturally occurring side chains of the 20 genetically encoded amino acids (or D amino acids) with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclic. In particular, proline analogs in which the ring size of the proline residue is changed from 5 members to 4, 6, or 7 members can be employed. Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic.

For example, the following peptides were prepared where "Na" or "Nap" represents naphthylalanine and "J" or "Aze" represents azetidine.

Table 24

Peptide	IC_{50}
SHLY-Na-QPYSVQM	+
TLVY-Na-QPYSLQT	+
RGDY-Na-QPYSVQS	+
NMVY-Na-QPYSIQT	+
VYWQPYSVQ	+
VY-Na-QPYSVQ	+
TFVYWQJYALPL	+

Other preferred peptides of the invention synthesized by techniques standard in the art are shown below.

Table 25

Peptide
VYWQPYSVQ
RLVYWQPYSVQR
RLVY-Nap-QPYSVQR
RLDYWQPYSVQR
RLVWFQPYSVQR
RLVYWQPYSIQR

The present invention also includes the peptides of the invention which have been modified, including, e.g., truncations, deletions, analogs, acylated and/or amidated derivatives, and the like. For example, the peptides may be truncated at either the amino or carboxy terminus, or both. Additionally, although the peptides have been described in terms of having a free carboxy and/or amino terminus, it may also be appreciated that one can modify the amino and/or carboxy terminus of the peptide compounds of the invention to produce other compounds of the invention.

For example, amino terminus modifications include methylating (i.e., $-NHCH_3$ or $-NH(CH_3)_2$), acetylating, adding a carbobenzoyl group, or blocking the amino terminus with any blocking group containing a carboxylate functionality defined by RCOO-, where R is selected from the group consisting of naphthyl, acridinyl, steroidyl, and similar groups. Carboxy terminus modifications include replacing the free acid with a carboxamide group or forming a cyclic lactam at the carboxy terminus to introduce structural constraints. One can also cyclize the peptides of the invention, or incorporate a desamino or descarboxy residue at the terminii of the peptide, so that there is no terminal amino or carboxyl group, to decrease susceptibility to proteases or to restrict the conformation of the peptide. C-terminal functional groups of the compounds of the present invention include amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, and carboxy, and the lower ester derivatives thereof, and the pharmaceutically acceptable salts thereof.

One can also readily modify peptides by phosphorylation, and other methods for making peptide derivatives of the compounds of the present invention are described in Hruby *et al.*, (1990) *Biochem J.* 268(2):249-262, incorporated herein by reference. Thus, the peptide compounds of the invention also serve as structural models for non-peptidic compounds with similar biological activity. Those of skill in the art recognize that a variety of techniques are available for constructing compounds with the same or similar desired biological activity as the lead peptide compound but with more favorable activity than the lead with respect to solubility, stability, and susceptibility to hydrolysis and proteolysis. *See* Morgan and Gainor (1989) *Ann. Rep. Med. Chem.* 24:243-252, incorporated herein by reference. These techniques include replacing the peptide backbone with a backbone composed of phosphonates, amidates, carbamates, sulfonamides, secondary amines, and N-methylamino acids.

Using the "very large scale immobilized polymer synthesis" system described in U.S. patent application Serial Nos. 492,462, filed March 7, 1990; 624,120, filed December 6, 1990; and 805,727, filed December 6, 1991; one can not only determine the minimum size of a peptide with such activity, one can also make all of the peptides that form the group of peptides that differ from the preferred motif (or the minimum size of that motif) in one, two, or more residues. This collection of peptides can then be screened for ability to bind to IL-1RtI. This immobilized polymer synthesis system or other peptide synthesis methods can also be used to synthesize every truncation analog and every deletion analog and every combination of truncation and deletion analog of all of the peptide compounds of the invention.

IV. *In Vitro* Uses

The compounds of the invention are useful *in vitro* as unique tools for understanding the biological role of IL-1, including the evaluation of the many factors thought to influence, and be influenced by, the production of IL-1 and

the receptor binding process. The present compounds are also useful in the development of other compounds that bind to the IL-1RtI, because the present compounds provide important information on the relationship between structure and activity that should facilitate such development.

The compounds are also useful as competitive inhibitors in assays to screen for new IL-1 receptor blockers. Specifically, the present invention includes peptides which bind the IL-1RtI, and wherein that binding is competitively inhibited by other peptides of the invention, e.g., those having the core sequences described herein. In such assay embodiments, the compounds of the invention can be used without modification or can be modified in a variety of ways; for example, by labeling, such as covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the materials thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups such as: radiolabels such as ^{125}I, enzymes (US Patent 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (US Patent 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups. The compounds may also include spacers or linkers in cases where the compounds are to be attached to a solid support. Methods of determining whether a compound competitively inhibits IL-1 receptor binding of a peptide of the invention are well known in the art. See, e.g. Stryer, Biochemistry, W.H Freeman and Co. (1988).

The compounds of the invention can also be used in assays as probes for determining the expression of the IL-1RtI on the surface of cells. Such an assay is useful for determining the degree of cellular immunological and inflammatory response, for example to infection and tissue injury. Typically, the cells under study will be exposed to the compounds for a period sufficient for the compounds to

bind to the receptor(s) exposed on the cell surface. The cells are then separated from the non-bound compounds and unreacted cells, *e.g.*, by affinity chromatography or the use of a cell sorter, to identify whether binding of the compounds to the receptor has occurred.

Thus, the compositions and methods of the present invention also can be used *in vitro* for testing a patient's susceptibility to varying treatment regimens for disorders associated with the overproduction of IL-1 or an improper response to IL-1 using an *in vitro* diagnostic method whereby a specimen is taken from the patient and is treated with a IL-1RtI binding, IL-1 blocking compound of the present invention to determine the effectiveness and amount of the compound necessary to produce the desired effect. The blocking compound and dosage can be varied. After the blocking compounds are screened, then the appropriate treatment and dosage can be selected by the physician and administered to the patient based upon the results. Therefore, this invention also contemplates use of a blocking compound of this invention in a variety of diagnostic kits and assay methods.

V. *In Vivo* Uses

The compounds of the invention can also be administered to warm blooded animals, including humans, to block the binding of IL-1α or IL-1β to the IL-1RtI *in vivo*. Thus, the present invention encompasses methods for therapeutic treatment of IL-1 related disorders that comprise administering a compound of the invention in amounts sufficient to block or inhibit the binding of IL-1 to the IL-1R *in vivo*. For example, the peptides and compounds of the invention can be administered to treat symptoms related to the overproduction of IL-1 or an improper response to IL-1. Since the biological effects of IL-1 include immunologic properties, such as T-cell activation, increased IL-2R expression, B-cell activation via induction of IL-6, natural killer cell activity, and lymphokine gene expression; pro-inflammatory properties such as fever, sleep, anorexia, neuropeptide

release, gene expression for complement, suppression of P450 synthesis, endothelial cell activation, neutrophilia, increased adhesion molecule expression, neutrophil priming, eosinophil degranulation, hypotension, myocardial suppression, neutrophil tissue infiltration, beta islet cell cytotoxicity, hyperlipidemia, cyclooxygenase and lipoxygenase gene expression, synthesis of collagenases and collagens, and osteoblast activation, the compositions and methods described herein will find use for the treatment and/or prevention of a variety of IL-1 related disorders. *See, e.g.*, Dinarello (1991) *Blood* 8:1627-1652, which is incorporated herein by reference. Examples of specific disorders having such symptoms include but are not limited to, atherlerosclerosis, rheumatoid arthritis, osteoporosis, HIV infection and AIDS, bacterial infection, respiratory distress syndrome, acute myelogenous leukemia ("AML"), graft vs. host disease, coal miner pneumonoconiosis, alcoholic cirrhosis, cuprophane hemodialysis, cardiopulmonary bypass, chronic hepatitis B, thermal injury, reticulohistiocytosis, sarcoidosis, tuberculosis, obstructive jaundice, Paget's disease and osteomalacia, IDDM, Kawasaki's disease, inflammatory bowel disease, sepsis, toxic shock, and luteal phase.

Accordingly, the present invention includes pharmaceutical compositions comprising, as an active ingredient, at least one of the peptides or other compounds of the invention in association with a pharmaceutical carrier or diluent. The compounds of this invention can be administered by oral, parental (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), nasal, vaginal, rectal, or sublingual routes of administration and can be formulated in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than inert diluents, *e.g.*, lubricating agents such as

magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

Preparations according to this invention for parental administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

The quantities of the IL-1 blocking compound necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for in

situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, *e.g.*, in Gilman et al. (eds), (1990) *Goodman and Gilman's: The Pharmacological Basis of Therapeutics*, 8th ed., Pergamon Press; and *Remington's Pharmaceutical Sciences*, (1985) 7th ed., Mack Publishing Co., Easton, Penn.; each of which is hereby incorporated by reference.

The dosage of active ingredient in the compositions of this invention may be varied; however, it is necessary that the amount of the active ingredient shall be such that a suitable dosage form is obtained. For the IL-1 blocking compounds of the invention exhibiting high affinity binding with IL-1 receptor, low dosages would be initially expected to be effective. Thus, generally dosage levels of between about 0.001 and 10 mg/kg, preferably between about 0.01 to 10 mg/kg, and more preferably between about 0.1 and 10 mg/kg of body weight daily will be administered to mammals to obtain effective IL-1 blocking activity.

It should, of course, be understood that the compositions and methods of this invention can be used in combination with other agents exhibiting the ability to modulate IL-1 synthesis, release, and/or binding. Examples of such agents include, but are not limited to disease modifying antirheumatic drugs chloroquine, auranofin, sodium aurothiomalate, and dexamethasone (*see, e.g.*, Lee *et al.* (1988) *Proc. Natl. Acad. Sci.* 85:1204); tenidap (*see, e.g.*, Otterness, 3rd Interscience World Conference on Inflammation, Monte-Carlo, Abstr. p. 371 (March, 1989); antioxidants, such as nordihydroguaiaretic acid (*see, e.g.*, Lee *et al.* (1988) *Int. J. Immunopharmacol.* 10:835), probucol (*see, e.g.*, Ku *et al.* (1988) *Am. J. Cardiol.* 62:778), and disulfiram (*see, e.g.*, Marx (1988) *Science* 239:257); pentoxifylline (*see, e.g.*, Sullivan *et al.* (1988) *Infect. Immun.* 56:1722); denbufylline (*see, e.g.*, Mandell *et al.* PCT publication WO 89/015145 (1989); romazarit (*see, e.g.*, Machin *et al.* (1988) U.S. Patent No. 4,774,253); tiaprofenic acid; dexamethasone; and natural

macromolecular IL-1 inhibitors (*see, e.g.*, Rosenstreich *et al.* in "*Lymphokines*", E. Pick, Ed., 14:6 Academic Press (1987) and Larrick (1989) *Immunol. Today* 10:6); as well as the other agents described in Bender and Lee (1989) *Annual Reports in Medicinal Chemistry Chapter 20: Pharmacological Modulation of Interleukin-1*, pp. 185-193, which is incorporated herein by reference.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight.

As can be appreciated from the disclosure above, the present invention has a wide variety of applications. Accordingly, the following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1

Solid Phase Peptide Synthesis

Various peptides of the invention were synthesized using Merrifield solid phase synthesis (*see* Stewart, J.M., and Young, J.D., Solid Phase Peptide Synthesis, 2d. edition (Pierce Chemical, Rockford, IL, 1984)) on a Milligen/Biosearch 9600 automated instrument. The resin used was PAL (Milligen/Biosearch), which is cross-linked polystyrene with 5-(4'-Fmoc-aminomethyl-3,5'-dimethoxyphenoxy) valeric acid as a linker. Use of PAL resin results in a carboxyl terminal

amide function upon cleavage of the peptide from the resin. Primary amine protection on amino acids was achieved with F-moc, and side chain protection groups were t-butyl for serine and tyrosine hydroxyls, trityl for glutamine amides, and Pmc (2,2,5,7,8-pentamethylchroman sulfonate) for the arginine guanidino group. Each coupling was performed for either one or two hours with BOP (benzotriazolyl N-oxtrisdimethylaminophosphonium hexafluorophosphate) and HOBt (1-hydroxybenztriazole).

Peptides having an amidated carboxy terminus were cleaved with a mixture of 90% trifluoroacetic acid, 5% ethanedithiol, and 5% water, initially at 4C, and gradually increasing to room temperature over 1.5 hours. The deprotected product was filtered from the resin and precipitated with diethyl ether. After thorough drying the product was purified by C18 reverse phase high performance liquid chromatography with a gradient of acetonitrile/water in 0.1% trifluoroacetic acid. Two peaks were isolated. The peptide in each peak was characterized by amino acid analysis and Fab mass spectrometry.

In the synthesis of the T6 peptide, with an amidated carboxy terminus, the fully assembled 12-mer was cleaved with a mixture of 90% trifluoroacetic acid, 5% ethanedithiol, and 5% water, initially at 4°C, and gradually increasing to room temperature over 1.5 hours. The deprotected product was filtered from the resin and precipitated with diethyl ether. After thorough drying the product was purified by C18 reverse phase high performance liquid chromatography with a gradient of acetonitrile/water in 0.1% trifluoroacetic acid. Two peaks were isolated. The peptide in each peak was characterized by amino acid analysis and Fab mass spectrometry. The mass spectrometry analysis is shown in Figure 1, parts A and B. These results show that a peak with the expected molecular weight (1594 daltons, part A) was produced; other results show that the other major peak (1795 daltons, part B) does not bind to IL-1R.

Peptide H_2N-trp-trp-thr-asp-asp-gly-leu-trp-ala-

ser-gly-ser-CO-NH_2 (the sequence in one letter abbreviation is WWTDDGLWASGS, although this designation does not show the carboxyl terminal amide function), which contains the peptide from fusion protein R11 (*see* Table 1, above), was synthesized according to the above procedure. The "ASGS" tetrapeptide was added to the carboxy terminal end of the peptide portion of fusion protein R11 to increase the solubility of the free peptide relative to a free peptide with the R11 peptide sequence alone. The synthetic peptide was determined to have the correct molecular weight by Fab mass spectroscopy.

Peptide H_2N-asp-trp-asp-gln-phe-gly-leu-trp-arg-gly-ala-ala-CO-NH_2 (DWDQFGLWRGAA), which is the peptide from fusion protein S14 (*see* Table 1, above), was synthesized on an Advanced Chemtech Model 350 peptide synthesizer using similar resins and F-moc protected amino acids. The activation of individual amino acids was by diisopropylcarbodiimide rather than BOP, and each thirty minute coupling was repeated. The cleavage reaction was performed in a mixture of 375 mg of phenol, 125 mL of ethanedithiol, 250 mL of thioanisole, 250 mL of deionized water, and concentrated trifluoroacetic acid to a total volume of 5 mL. The mixture was initially incubated at 4C and then warmed gradually to room temperature over 1.5 hours. Filtering, precipitation, and purification were performed as described above.

Peptide H_2N-tyr-trp-asp-thr-arg-gly-leu-trp-val-tyr-thr-ile-CO-NH_2 (the sequence in one letter abbreviation is YWDTRGLWVYTI), which is the peptide from fusion protein S4, except for the substitution of Y for W outside the WXXXG(I/L)W motif (*see* Table 1, above), was likewise synthesized on the Chemtech Model 350 according to the above procedure. The substitutions increase the solubility of the free peptide relative to the S4 sequence alone.

EXAMPLE 2

A. Prostaglandin E_2 (PGE_2) Response Assay

Some of the peptides were tested for ability to block the IL-1α induced PGE_2 response in human foreskin fibroblasts. IL-1α is known to stimulate the production of

PGE_2 by normal human fibroblasts, and an assay for PGE_2 is available from Amersham (*see* the instruction manual for the Prostaglandin $E_2[^{125}I]$ scintillation proximity assay (SPA) system, code RPA 539, incorporated herein by reference. Except as otherwise noted below, the assay was carried out according to the manufacturer's instructions and with the reagents supplied in the kit.

For use in the assay, the cells should be actively growing at near confluence in 96 well tissue culture plates. Typically, cells were kept at about 60% confluency under "starved" conditions (low serum in the media) until needed for the assay. One day before the assay, as many wells as needed for the assay were seeded with about 1.5×10^4 cells per well in 100 µl of DMEM/F12 (a 1:1 mixture of the two medias, supplied by JRH Biosciences) plus 10% fetal bovine serum (FBS, supplied by Hyclone) and antibiotics (penicillin and streptomycin, P/S) to allow the cells to begin active growth.

Each peptide in powder form was individually dissolved in DMEM/F12 containing 1% FBS and P/S to a final concentration of about 675 µM. 12 wells of a microtiter dish were typically used to assay each peptide. The media was removed from the cells (the cells were not washed) with a pipettor, and 140 µl of the peptide solution were added to half of the wells (i.e, peptide was added to a set of 6 wells and the other set of 6 wells served as the control), and the plate was incubated for 30 minutes at 37°C. Then, 10 µl of a 15 pM solution of IL-1α (in the same DMEM solution as the peptide) were added to half of the wells containing peptide (i.e., typically 3 wells) and half of the wells with no peptide (i.e., 3 wells). About 10 µl of media were added to the wells which did not contain the IL-1α solution, and the plate was incubated for 6 hours at 37°C. Thus, for each peptide, the 3 wells of the microtiter dish contained no peptide and no IL-1α; 3 wells contained no peptide and IL-1α; 3 wells contained peptide and no IL-1α; and 3 wells contained peptide and IL-1α.

About 100 µl of media from each microtiter well (the sample) were added to 100 µl of the methyl oximation reagent,

and the resulting mixture was incubated at 60°C for one hour. About 300 µl of assay buffer were then added to each sample, and the sample was vortexed to ensure complete mixing. About 100 µl of the resulting sample were added to a labeled 1.5 ml Eppendorf™ tube. About 200 µl of assay buffer were added to two tubes labelled NSB (non-specific binding). About 100 µl of assay buffer were added to two tubes labelled B_0 (B_0 is equal to the number of counts (cpm of ^{125}I) bound to the bead in the absence of PGE_2). About 100 µl of tracer (^{125}I-PGE_2-methyloximate; the methyloximate derivative of PGE_2 is more stable than PGE_2) are added to each tube, and 100 µl of anti-methyloximate PGE_2 antisera were added to all tubes except the NSB tubes. Then, about 100 µl of beads, which contain the scintillant and are coated with Protein A, were added to each tube.

The Protein A binds to the antisera, and the antisera binds to the ^{125}I-PGE_2-methyloximate. Unlabeled PGE_2, the production of which is stimulated by IL-1α, competes with the ^{125}I-PGE_2-methyloximate, thus reducing the total ^{125}I cpm bound to the bead. PGE_2 concentration was calculated from a standard curve as described in the Amersham PGE_2 SPA assay instruction manual. The results indicated that at least some of the peptides did inhibit the PGE_2 response to 1 pM IL-1 after 3 hours and 6 hours incubation.

B. Epidermal Growth Factor Receptor Down Regulation Assay

The protocol set forth in Bird and Saklatvala (1989) *J. Immunol.* 142:126-133, which is incorporated herein by reference, was also used to demonstrate the activity of the compounds of the present invention. This assay examines the effect of the compounds of the invention on the IL-1 induced down-regulation of the epidermal growth factor (EGF) receptor as measured by the binding of ^{125}I-labeled EGF to its receptor in normal human dermal fibroblasts (NHDF). Incubation of the cells with the compounds of the present invention at 37°C, in the presence of IL-1α showed no decrease in the cells ability to subsequently bind subsaturating levels of ^{125}I-labeled

epidermal growth factor, whereas incubation in the presence of IL-1α alone, resulted in a decrease in EGF binding. The table below lists the approximate affinity of representative peptides of the invention. The affinity is determined as the concentration of peptide which blocks 50% of the IL-1 induced down regulation of the EGF receptor.

Table 26

Peptide	IC_{50}
ETPFTWEESNAYYWQPYALPL	++
TFVYWQJYALPL	-
TFvYWQPYALPL[2]	-
YIPFTWEESNAYYWQPYALPL	++
DGYDRWRQSGERYWQPYALPL	++
TANVSSFEWTPGYWQPYALPL	++
FEWTPGYWQJYALPL	++
FEWTPGYWQPYALPLSD-NH_2*	++
FEWTPGYYQJYALPL	++

* "NH_2" denotes an amide group, e.g. a carboxyamide.

C. Peptide Effects on IL-1 Induced E-Selectin (HUVEC Cells)

The compounds of the present invention were assayed for their ability to inhibit the expression of IL-1 induced E-selectin in HUVEC cells (human umbilical vein endothelial cells).

HUVEC cells are grown to confluency in the wells of a 24-well microtiter plate. The compounds were serially diluted in DMEM/F12 + 0.1 % BSA ("SFM") with 120 pM IL-1α. The cells were washed and then incubated with IL-1α and the compound to be tested for 15 minutes. The cells were again washed and incubated in SFM for 4 hours. The cells were washed and incubated with anti-selectin antibody for 30 minutes. Following antibody incubation, the cells were incubated with ^{125}I Protein A and washed. The amount of ^{125}I

[2] d-valine is used at the 3-position.

bound to the cells was then determined. The concentration of compounds required to inhibit 50% of IL-1 induced E-selectin expression (IC_{50}) is shown below.

Table 27

Peptide	IC_{50}
FEWTPGYYQJYALPL	++
FEWTPGYWQJYALPL	++
FEWTPGWYQJYALPL	++
TANVSSFEWTPGYWQPYALPL	++

D. Other Assays

Other biological assays that can be used to demonstrate the activity of the compounds of the present invention are disclosed in Dripps, *et al.*, (1991) *J. Biol. Chem.* 266(16):10331-10336, and Bird, *et al.*(1991) *J. Biol. Chem.* 266(33):22662-22670, each of which is incorporated herein by reference.

EXAMPLE 3

Determination of Concentration Effecting Inhibition of IL-1α Binding

This example provides the results of experiments conducted to determine an approximate IC_{50} for certain peptides of the invention. IC_{50} as used herein refers to the concentration of a compound which will inhibit 50 % of the binding between a receptor and its ligand, e.g. IL-1RtI and IL-1. Stock solutions of each peptide were prepared. The appropriate amount of the peptide was dissolved in DMSO, and then nineteen volumes of binding buffer (RPMI 1640, 1% BSA, 20 mM HEPES, pH 7.2-7.3, and 0.1% sodium azide) were added to yield a 1 mM peptide, 5% DMSO stock solution.

One assay utilized a truncated IL-1RtI which had been immobilized on 96-well plates with an appropriate antibody, typically a non-blocking high affinity antibody. In other assays, cells expressing "full-length" IL-1RtI were used with results that yielded IC_{50} values about 5 to 10 fold lower than those determined using the truncated receptor. The cells

were seeded onto Falcon 3072 96-well plates at about 10^5 cells per well, and the plates were incubated overnight at 37°C in media containing serum. The following morning, the cells were checked to ensure that the cells were confluent and adhered to the bottom of the wells.

According to either assay protocol, the plates were then washed three times with binding buffer, and then 50 μl of binding buffer and 25 μl of a peptide solution (either the stock solution or a dilution thereof; each stock was subjected to five three fold dilutions) were added to each well. Then 25 μl of binding buffer containing ^{125}I-IL-1α (final concentration of 90 pM) were added to each well to begin the assay. Each assay was carried out in duplicate. The plates were then incubated for two hours at 4°C. For peptides having particularly high affinities, the assay was run in 24 well plates allowing for higher levels of ligand (possible total volume of 2.5 ml instead of 100 μl in the 96 well plate), thereby minimizing the potential for ligand depletion.

After the two hour incubation, the wells were rinsed three times with ice cold PBS (a semi-automated cell harvesting device was used to conduct the rinse). The receptors or cells were then detached from the plates by adding 100 μl of 0.1 N NaOH to each well and incubating the plates at room temperature for 20 minutes. After the 20 minute incubation, about 75 μl of the suspension was counted on a gamma counter, and the IC_{50} for each peptide was determined using computer assistance and the results of the gamma counting.

WE CLAIM:

1. A compound that binds to an interleukin-1 type I receptor, said compound comprising

a molecular weight less than about 3000 Daltons;

a binding affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 2.5 mM; and

wherein the binding of said compound to said interleukin-1 type I receptor is competitively inhibited by a peptide of eight to forty amino acids in length, said peptide comprising a core sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; and Z_6 is S, A, V, or L.

2. The compound of claim 1, wherein said compound comprises an affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 100 μM.

3. The compound of claim 1, wherein said compound comprises an affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 1 μM.

4. The compound of claim 1, wherein said compound blocks binding of interleukin-1 to an interleukin-1 type I receptor.

5. The compound of claim 1, wherein said compound is a peptide.

6. A compound that binds to an interleukin-1 type I receptor, said compound comprising

a molecular weight less than about 3000 Daltons;

a binding affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 2.5 mM; and

wherein the binding of said compound to said interleukin-1 type I receptor is competitively inhibited by a peptide of eight to forty amino acids in length, said peptide comprising a core sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; and Z_6 is S, A, V, or L.

7. The compound of claim 6, wherein said compound comprises an affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 100 μM.

8. The compound of claim 6, wherein said compound comprises an affinity to an interleukin-1 type I receptor as expressed by an IC_{50} standard of no more than about 1 μM.

9. The compound of claim 6, wherein said compound blocks binding of interleukin-1 to an interleukin-1 type I receptor.

10. The compound of claim 6, wherein said compound is a peptide.

11. A compound of seven to forty amino acids in length that binds to interleukin-1 type I receptor and comprises a sequence of amino acids $WXXXGZ_1W$ where each amino acid is indicated by standard one letter abbreviation; each X can be selected from any one of the 20 genetically coded L-amino acids; and Z_1 is L, I, A, or Q.

12. The compound of claim 11 where the sequence of amino acids comprises WZ_2XXGZ_1W where X can be selected from any one of the 20 genetically coded L-amino acids; Z_1 is L, I, A, or Q; and Z_2 is D, G, N, S, or T.

13. The compound of claim 12 where the sequence of amino acids comprises $WZ_2Z_3Z_4GZ_1W$ where Z_1 is L, I, A, or Q; Z_2 is D,

G, N, S, or T; Z_3, is D, E, H, M, N, Q, R, S, T, or V; and Z_4 is A, D, F, H, K, N, Q, R, T, or Y.

14. The compound of claim 11 where the sequence of amino acids comprises $WZ_2Z_3Z_4GZ_1W$ where Z_1 is L or I; Z_2 is D, S, or T; Z_3 is D, E, or T; and Z_4 is D, H, N, R, or T.

15. The compound of claim 11 that is 7 to 25 amino acid residues in length.

16. The compound of claim 11 having the sequence SWDTRGLWVE.

17. A compound of eight to forty amino acids in length that binds to interleukin-1 type I receptor and comprises a sequence of amino acids $XXQZ_5YZ_6XX$ where X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; and Z_6 is S, A, V, or L.

18. The compound of claim 17 where the sequence of amino acids comprises $Z_7XQZ_5YZ_6XX$ where each amino acid is indicated by standard one letter abbreviation; X can be selected from any one of the 20 genetically coded L-amino acids; Z_5 is P or Aze where Aze is azetidine; Z_6 is S or A; and Z_7 is Y, W, or F.

19. The compound of claim 18 where the sequence of amino acids comprises $Z_7Z_8QZ_5YZ_6Z_9Z_{10}$ where Z_5 is P or Aze where Aze is azetidine; Z_6 is S or A; Z_7 is Y, W, or F; Z_8 is E, F, V, W, or Y; Z_9 is M, F, V, R, Q, K, T,S, D, L, I, or E; and Z_{10} is E, L, W, V, H, I, G, A, D, L, Y, N, Q or P.

20. The compound of claim 19 where the sequence of amino acids comprises $Z_7Z_8QZ_5YZ_6Z_9Z_{10}$ where Z_5 is P or Aze where Aze is azetidine; Z_6 is S or A; Z_7 is Y, W, or F; Z_8 is E, F, V, W, or Y; Z_9 is V, L, I, or E; and Z_{10} is Q or P.

21. The compound of claim 20 where the sequence of amino acids comprises $Z_{11}Z_7Z_8QZ_5YZ_6Z_9Z_{10}$ where Z_{11} is V, L, I, E, P, G, Y, M, T, or D.

22. The compound of claim 21 where the sequence of amino acids is selected from the group consisting of TANVSSFEWTPGYWQPYALPL; SWTDYGYWQPYALPISGL; ETPFTWEESNAYYWQPYALPL; ENTYSPNWADSMYWQPYALPL; SVGEDHNFWTSEYWQPYALPL; DGYDRWRQSGERYWQPYALPL; FEWTPGYWQPYALPL; FEWTPGYWQJYALPL; FEWTPGYYQPYALPL; FEWTPGYYQJYALPL; FEWTPGWYQPYALPL and FEWTPGWYQJYALPL.

23. The compound of claim 17 that is 8 to 25 amino acid residues in length.

24. The compound of claim 1, 6, 11 or 12 that is conjugated to a cytotoxic agent.

25. A pharmaceutical composition comprising a compound of claim 1, 6, 11 or 12 in combination with a pharmaceutically acceptable carrier.

26. A method for treating a patient having a disorder that is susceptible to treatment with a IL-1 inhibitor wherein the patient receives, or is administered, a therapeutically effective dose or amount of a compound of claim 1, 6, 11 or 12.

27. A method of antagonizing action of Interleukin-1 on an interleukin-1 type I receptor, said method comprising contacting said receptor with a compound of claim 1, 6, 11 or 12.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01590

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07K 5/00, 7/00, 14/00, 17/00.
US CL :514/16, 15, 14, 13, 12; 530/324, 325, 326, 327, 328.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/16, 15, 14, 13, 12; 530/324, 325, 326, 327, 328.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 4,968,607 (DOWER ET AL.) 06 NOVEMBER 1990, SEE ENTIRE DOCUMENT.	1-27
Y	US, A, 5,039,790 (ADAMS ET AL.) 13 AUGUST 1991, SEE ENTIRE DOCUMENT.	1-27
Y	US, A, 5,077,219 (AURON ET AL.) 31 DECEMBER 1991, SEE ENTIRE DOCUMENT.	1-27
Y	US, A, 5,075,222 (HANNUM ET AL.) 24 DECEMBER 1991, SEE ENTIRE DOCUMENT.	1-27
Y	US, A, 5,075,288 (KRUEGER ET AL) 24 DECEMBER 1991, SEE ENTIRE DOCUMENTS.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

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